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Comparison between Fermentation and Ultrasound-Assisted Extraction: Which Is the Most Efficient Method to Obtain Antioxidant Polyphenols from *Sambucus nigra* and *Punica granatum* Fruits?

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Abstract: Fruit extracts of *Sambucus nigra* L. (elderberry) and *Punica granatum* L. (pomegranate) have several applications in nutraceutical, cosmetics, and pharmaceutical industries thanks to their richness in antioxidant polyphenols, whose composition changes with the extraction method applied. We aimed to compare the efficiency of the fermentation extraction, recently applied by industries, with the ultrasound-assisted extraction–UAE, a well-known and efficient technique, on the yield of antioxidant polyphenols from elderberry fruits and pomegranate fruit-peels. Extracts were obtained by both methods, analyzed by high-performance liquid chromatography (HPLC) and the antioxidant capacities were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Hydroxyl Radical Scavenging (HRS) assays. The main compounds detected in elderberry were caffeoyl and quercetin derivatives, present in higher amounts in UAE extracts. In pomegranate, punicalagin were the main constituents, also detected in higher contents in the UAE extracts compared to fermented ones. The UAE was more suitable for extracting anthocyanins from pomegranate. In addition, higher antioxidant capacities were observed in UAE extracts, possibly due to their richness in polyphenols. Therefore, despite the recent wide applicability and the good performance of the fermentation process, the UAE may be considered more efficient for the extraction of polyphenols from *S. nigra* and *P. granatum* fruits and may be used to obtain polyphenolic antioxidant extracts to be applied by several industries.

Keywords: anthocyanins; DPPH; elderberry; fermentation; flavonoids; HRS; LC-ESI-Q-ToF; pomegranate peels; UAE

1. Introduction

Fruit is a rich source of bioactive compounds and has been explored for direct or indirect applications by food, pharmaceutical, and cosmetic industries, to obtain several products [1,2]. Among the compounds present in this plant material, polyphenols have noticeable importance [3].

In plants, polyphenols play several protective and ecological roles, and, in fruit, they act especially as colorants and flavoring agents to attract seed dispersers [4]. Regarding their health-related properties, polyphenols are important for the prevention of chronic and degenerative diseases, particularly related to oxidative stress, including cardiovascular

disorders, diabetes, and cancer [3,5]. Indeed, the high antioxidant potential of polyphenols undoubtedly explains their bioactivity [3,4]. Due also to their anti-inflammatory, immunomodulatory and antiseptic actions, phenolic compounds are ideals preventive and healing agents [2,6]. Synthetic antioxidants are being replaced by natural polyphenolic-rich extracts in several products [7–9].

Berries and red fruits are some of the main sources of polyphenolic compounds used as cosmeceuticals [6,10] and have already shown multiple health benefits [11–13]. Among them, elderberry (*Sambucus nigra* L.) and pomegranate (*Punica granatum* L.) have remarkable commercial importance. Indeed, several products using elderberry and pomegranate extracts are currently available, and these fruits are among the main sources of polyphenols and colorants used by cosmetic and pharmaceutical industries [6,14].

Sambucus nigra L. (Adoxaceae) is a deciduous tree-like shrub widespread in Europe, especially in Mediterranean areas. Traditionally, the fruits of this species are used for the production of pies, jellies, jams, and beverages [15–17]. In addition, the fruit extracts have been applied as a diuretic and antiviral [14,17,18] and have been demonstrated anti-inflammatory, antidiabetic and immune-stimulating activities, some of them attributed to their high polyphenolic content [14,16]. Moreover, elderberry extracts have also shown high antioxidant capacity [14,16,19] and potential amelioration of UVB-induced skin photoaging [20].

Punica granatum L. (Lythraceae) fruits are widely consumed around the world. Despite being native to Asia, pomegranate cultivation and consumption have been spread in the Mediterranean basin, including in Spain and Italy [21,22]. Extracts of pomegranate have shown anti-inflammatory and anti-cancer properties [23,24], which might be attributed to their well-known prominent antioxidant activity. In addition, pomegranate peel extracts have shown hypolipemic and hypoglycemic effects in humans with type 2 diabetes [25]. Moreover, pomegranate extracts are used as bioactive constituents in cosmetic products for the treatment of wrinkles and promotion of skin repair [6].

The main parts of pomegranate fruits are the peels (sum of the mesocarp, with white color, and exocarp, with orange-to-red color), and the seeds, these last covered by the juice-sacs [26]. All of these contain interesting bioactive molecules; however, the peels represent more than 40% of the fruit's fresh weight and are rich in polyphenols, particularly in hydrolyzable tannins, even more than the pulp and the seeds [27]. Thus, some medicines have been currently produced using peels, a common waste material with large annual production [22,26].

The polyphenolic composition of an extract depends on the method of extraction [28]. Indeed, polyphenols are found in different plant cell structures and as insoluble forms through ester, ether, or glycosidic bonds, which turn their extraction a challenge [29]. Therefore, industries are constantly looking for more effective extraction processes to efficiently recover these bioactive compounds [6,29].

Ultrasound-assisted extraction (UAE) is among the most efficient extraction processes for recovering polyphenols from fruit, vegetables, herbs, and spices [8]. It is considered a key technology based on the breakdown of the plant material by means of ultrasonic cavitation phenomena, being a simple, highly effective, and low-cost extraction technique [8,30]. UAE is widely applied in the pharmaceutical and cosmetic fields of the 21st century [31].

Fermentation, on the other hand, has been recently employed to extract polyphenols from several plant materials, standing out as a useful technology to produce novel cosmeceutical and pharmaceutical agents [32–34]. It involves enzyme-catalyzed reactions and induces the breakdown of the plant tissues, generally applying water as a solvent. Fermentation is based on the action of different ligninolytic and carbohydrate-metabolizing enzymes as well as microorganisms, which facilitate the extraction of polyphenols and other several compounds. The use of fermentation as an extraction method can increase the content of bioactive compounds, also by chemical changes of the original molecules, for example releasing free aglycones [32–35]. In particular, the application of *S. cerevisiae* is reported as capable to affect the hydrolysis of ellagitannins leading to a higher yield

of these compounds in pomegranate husks [36]. In addition, this fermentation has been successfully applied to fruits containing anthocyanins [37,38]. Moreover, the combination of microbial fermentation with enzymatic treatment, in a simultaneous or sequentially way, has been recently applied to improve the content of polyphenols and the bioactivity of final products [39,40]. Due to its novelty and recently large application, the comparison between the efficiency of this extraction method with that of other techniques is of great importance, being barely found in the literature.

Although there are several reports regarding the polyphenolic composition of *S. nigra* and *P. granatum* fruit extracts, few of them compare the effects of different extraction methods in the yield of polyphenols and their antioxidant activity. Moreover, the comparison of the UAE, a well-known and efficient technique, largely applied for polyphenols extraction by industries, with fermented-assisted extraction, a recently employed method, has not been conducted yet. Therefore, this study aimed to compare the efficiency of the fermentation process (enzymatic treatment followed by incubation with *S. cerevisiae*) with that of UAE to achieve polyphenolic antioxidant extracts from the fruits of elderberry and pomegranate, two species largely applied by the cosmetic and pharmaceutical industries.

2. Materials and Methods

2.1. Fruit Material and Extraction Processes

Five three-years-old plants of *Sambucus nigra* L. and five plants of *Punica granatum* L., bought from a commercial nursery in Florence (Italy), were planted in pots filled with sandy soil (sand/peat, 60:40, *v*/*v*) and maintained in the greenhouse of the Department of Agriculture, Food, Environment and Forestry (DAGRI)—University of Florence (UNIFI), Sesto Fiorentino (Florence, Italy, 43°49′ N, 11°37′ E). At the fully ripened stage, three fruits per plant of *P. granatum* and three fruit clusters (cones) of *S. nigra* were collected, in August 2019 and October 2019, respectively.

For *S. nigra*, completely mature berries (dark-violet colors) were removed from the stem, immediately frozen at −80 °C, and lyophilized. For *P. granatum*, immediately after harvesting, the peels (exocarp + mesocarp) were separated from the rest of the fruit (endocarp + seeds), frozen (at −80 °C) and lyophilized. Two types of extraction were conducted with the dry materials, namely fermentation and ultrasonic-assisted extraction (UAE), according to the literature protocols [18,36,40,41].

For the enzymatic fermented extracts, 1 g of dry material was ground in liquid nitrogen, suspended in 15 mL of hot water (85 °C) for 15 min, treated with xylanase (1500 units), α-amylase (1000 units), and glucosidase (5.8 units) (all from Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany), and fermented at room temperature (28 °C) for 24 h using *Saccharomyces cerevisiae* (0.5% *w*/*v*, Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany). For the UAE-ethanolic extracts, the ground dry material (1 g) was extracted with 15 mL of ethanol 75% (pH 2.5 adjusted with formic acid–FA, both from Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany) by ultrasonic-assisted extraction (UAE) in ice-ultrasonic-bath (BioClass® CP104) using a constant frequency of 39 kHz and an input power of 100 W, for 30 min, at 5 °C. The lipophilic fraction was removed by liquid/liquid extraction with 15 mL *n*-hexane (Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany), to avoid interferences in the following analyses. After the extraction, both fermented and UAE-ethanolic extracts were dried in rotavapor, weighted, and resuspended in methanol: water (1:1) acid solution (pH 2.5, adjusted with formic acid) to reach a final concentration of 10 mg mL^{-1}. All the extractions were conducted in triplicate.

2.2. LC-ESI-Q-ToF Analysis: Identification of Polyphenols

After confirming by HPLC-DAD analysis that fermented and UAE extracts did not differ in terms of phenolic composition, but only in terms of concentration of the compounds, the fermented extracts were analyzed by LC-ESI-Q-ToF for more detailed identification of the different compounds.

For this analysis, the system consisted of an HPLC 1200 Infinity, coupled with a quadrupole-time of flight mass spectrometer Infinity Q-ToF 6530 detector by a Jet Stream ESI interface (Agilent Technologies). The ESI conditions were drying and sheath gas N_2, purity > 98%, temperature 350 °C, flow 10 L min^{-1} and temperature 375 °C, flow 11 L min^{-1}, respectively; capillary voltage 4.5 KV; nebulizer gas pressure 35 psi. The fragmentor voltage was 175 V; nozzle, skimmer, and octapole RF voltages were set at 1000 V, 65 V, and 750 V, respectively. The high-resolution MS and MS/MS acquisition range was set from 100 to 1000 m/z in both positive and negative mode, with an acquisition rate of 1.04 spectra/s. For the MS/MS experiments, 30 V were applied in the collision cell to obtain CID fragmentation (collision gas N_2, purity 99.999%). The FWHM (full width half maximum) of quadrupole mass bandpass used during MS/MS precursor isolation was 4 m/z. The Agilent tuning mix HP0321 was used daily to calibrate the mass axis.

The chromatographic separation was performed on an analytical reversed-phase column Poroshell 120 EC-C18 (3.0 × 75 mm, particle size 2.7 μm) with a Zorbax® pre-column (4.6 × 12.5 mm, particle size 5 μm), both by Agilent Technologies. The eluents used were water (A) and acetonitrile (B), both LC-MS grade (Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany), and added with 0.1% *v/v* FA (98% purity, J.T. Baker®, Milan, Italy).

Aliquots of the fermented extracts of both species (5 μL, 10 mg mL^{-1}) were analyzed using a flow rate of 0.4 mL min^{-1} and the program was: 15% B (0.1% FA in ACN) for 2.6 min, then to 50% B in 13.0 min, to 70% B in 5.2 min, to 100% B in 0.5 min and then held for 6.7 min, with are-equilibration of 11 min. During the separation, the column was kept at 30 °C. The identification of the polyphenols present in the different extracts was conducted based on the exact mass and tandem mass spectra obtained by the ESI-Q-ToF detector, by comparison with reference compounds (quercetin, kaempferol, caffeic, chlorogenic, gallic and ellagic acids, catechin and punicalagin, all from Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany) and literature data.

2.3. HPLC-DAD Analysis: Quantification of Polyphenols

To quantify the different classes of polyphenols (hydroxycinnamic acid derivatives, flavonoids, and anthocyanins), aliquots of all the extracts (5 μL, 10 mg mL^{-1}) were injected into a Perkin® Elmer Flexar liquid chromatograph equipped with a quaternary 200 Q/410 pump and an LC 200 diode array detector (DAD) (all from Perkin Elmer®, Bradford®, CT, USA). The stationary phase consisted of an Agilent®Zorbax® SR-18 (250 × 4.6 mm, 5 μm) column kept at 30 °C. The eluents were (A) acidified water (pH 2.5, 0.1% FA) and (B) acetonitrile (pH 2.5, 0.1% FA). A gradient solvent system from 98% of A to 98% of B, over a 44-min run and a flow rate of 0.6 mL min^{-1} was applied: 0–2 min (2% B), 2–37 min (2–98% B), 37–42 min (98% B), 42–44 min (98–2% B). The chromatograms were acquired at 280 nm and 350 nm.

For anthocyanins quantification, a Luna Omega® Phenomenex® C18 (100 × 2.1 mm, 3 μm) column was used. The same solvents were used at the same gradient, but with a flow rate of 0.4 mL min^{-1}. The chromatograms were acquired at 520 nm.

Different standards (punicalagin, chlorogenic acid, caffeic acid, quercetin, kaempferol, ellagic acid, and cyanin, all from Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany) were injected to generate five-point calibration curves (0.025–0.5 mg mL^{-1}) used to quantify the diverse phenolic classes of the compounds detected in the extracts. All constituents were quantified using these standards by the external standard method and the molecular weight correction factors were used when necessary. The linearity of the curves was determined by the coefficient of determination (R^2), being higher than 0.99 for all the standards. The results were given in milligrams to grams of dry weight (mg g^{-1} DW).

The content of different classes of polyphenols in *S. nigra* was given in total amounts of the main phenolic classes detected: total flavonoids (TFC), total hydroxycinnamic acid derivatives (THC), total anthocyanins (TAC), and total polyphenols content (TPC), all as the sum of the individual compounds belonging to each class and quantified with each

specific standard. For *P. granatum*, the amount of the major compounds (punicalagin alpha and beta), anthocyanins (TAC), and polyphenols (TPC) were determined.

2.4. Antioxidant Capacity Assays

The antioxidant capacity assay was performed using two different methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and the Hydroxyl Radical (OH)-Scavenging (HRS) assay.

The DPPH assay was performed according to the method described by Kandi and Charles [42]. Briefly, diluted samples of the extracts (0.5 mL) were added to 0.5 mL of DPPH solution (0.1 mM in methanol; Sigma–Aldrich®–Merck®KGaA, Darmstadt, Germany) and the mixture was kept reacting at room temperature for 45 min in the dark. After this time, the absorbance was spectrophotometrically measured at 518 nm, using a Perkin Elmer® UV/Vis spectrophotometer (Lambda 25). Aliquots of the extracts without the addition of the DPPH solution (0.5 mL methanol and 0.5 mL extracts) were used as blanks, and the DPPH solution was used as negative control (0.5 mL methanol and 0.5 mL DPPH solution). The analyses were conducted in triplicate and the percentage of antioxidant activity was calculated as follow (Equation (1)):

$$AA\% = 100 - \{[(ABS_{sample} - ABS_{blank}) \times 100]/ABS_{negative\ control}\} \quad (1)$$

The Hydroxyl Radical-Scavenging (HRS) assay was performed as described in Gori et al. [43]. Different concentrations of the extracts were allowed to react with $FeSO_4$ (1.5 mM), hydrogen peroxide (6 mM), and sodium salicylate (20 mM), all from Sigma–Aldrich®(Merck®KGaA, Darmstadt, Germany), at 37 °C for 1 h. Afterward, the absorbance was measured at 562 nm.

The EC_{50} (effective concentration at 50%) values were calculated with Microsoft Excel® software.

2.5. Statistical Analysis

The content of polyphenols and the antioxidant capacities (EC_{50} values) of the extracts were expressed as mean ± standard deviation (SD) (n = 3). A Student's t-test (factor: extraction method) was used to compare the results. All the statistical analyses were performed using SigmaPlot® Systat® software (version 12.5, SystatSoftware, Inc., San Jose, CA, USA) and the differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Polyphenolic and Anthocyanic Content in Fermented and UAE-Ethanolic Extracts

The main polyphenols detected in extracts of *S. nigra* fruits were hydroxycinnamic acid derivatives (especially caffeoylquinic and caffeic acids) and quercetin glycosides (flavonols) (Supplementary Figure S1, Table 1). These compounds were identified based on the HPLC-ESI-Q-ToF analysis, by comparison with reference compounds and literature data, as follows: neochlorogenic acid (peak 1), dihydroxybenzoic acid (peak 2), cryptochlorogenic acid (peak 3), chlorogenic acid (peak 4), caffeic acid (peak 5), quercetin-3-*O*-rutinoside (peak 6), quercetin-3-*O*-glucoside (peak 7), kaempferol-*O*-rutinoside (peak 8) and quercetin (peak 9) (Supplementary Figure S1, Table 1). The most abundant class of compounds in both extracts (Table 2) were flavonoids, among which rutin (peak 6) was the main constituent (Supplementary Figure S1).

Table 1. Peaks detected in extracts of fruits of *S. nigra* by HPLC-ESI-Q-ToF analysis (peak numbers are referred to the chromatograms presented in Supplementary Figure S1).

Peak	Tr (min)	Raw Formula	Molecular ion $[M-H]^-$ (*m/z*)	MS/MS Fragments	Peak Assignment
1	1.9	$C_{16}H_{18}O_9$	353.0893	191.0561, 179.0342, 135.0461	Neochlorogenic acid (5-*O*-caffeoylquinic acid)
2	2.1	$C_7H_6O_4$	153.1235	109.0421	Dihydroxybenzoic acid
3	2.4	$C_{16}H_{18}O_9$	353.0895	191.0572, 173.0451, 127.0410	Cryptochlorogenic acid (4-*O*-caffeoylquinic acid)
4	3.4	$C_{16}H_{18}O_9$	353.0884	191.0566	Chlorogenic acid (3-*O*-caffeoylquinic acid)
5	3.7	$C_9H_8O_4$	179.0354	135.0461	Caffeic acid
6	7.9	$C_{27}H_{30}O_{16}$	609.1484	300.0281, 151.0021	Quercetin-3-*O*-rutinoside (rutin)
7	8.6	$C_{21}H_{20}O_{12}$	463.0894	300.0278, 271.0258, 151.0035	Quercetin-3-*O*-glucoside
8	9.4	$C_{27}H_{30}O_{15}$	593.1534	285.0413	Kaempferol-3-*O*-rutinoside
9	13.1	$C_{15}H_{10}O_7$	301.0367	151.0040, 121.0293, 107.0139	Quercetin

Table 2. Content of polyphenols (in mg g^{-1} DW) in UAE-ethanolic and fermented extracts of elderberry (*S. nigra*) fruits. TFC—total flavonoid content; THC—total hydroxycinnamic acid derivatives content; TAC—total anthocyanins content; TPC—total polyphenols content.

S. nigra	TFC	THC	TAC	TPC
UAE-ethanolic extracts	2.95 ± 0.5 *	0.70 ± 0.08 ***	0.311 ± 0.05	3.96 ± 0.4 *
Fermented extracts	1.66 ± 0.25	0.22 ± 0.02	1.23 ± 0.32 **	3.11 ± 0.2

Results are given in mean ± SD (n = 3). Asterisks indicate significant differences between the content of the same compound class in different extracts (UAE vs. fermented), according to the student t-test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Regarding the amount of the different classes of polyphenols in both extract types, the UAE extracts resulted to be richer especially in THC ($p < 0.001$), being around three times higher than that content found in fermented extracts. The UAE was also more efficient in extracting flavonoids (TFC was around double higher, $p = 0.02$) and total polyphenols (TPC, $p = 0.03$) (Table 2). In contrast, anthocyanins (TAC) showed higher concentrations in fermented extracts compared to UAE ($p \leq 0.01$, Table 2). Indeed, the content of anthocyanins in fermented extracts is almost the same as flavonoids in these extracts (Table 2).

The polyphenolic composition of the extracts of *P. granatum* fruit-peels revealed the presence of punicalagin alpha (peak 3) and beta (peak 6) as the major constituents (Supplementary Figure S2, Table 3), both extracted in higher amounts by UAE–around 2 to 3 times more (Table 4, $p \leq 0.001$). Further, less abundant peaks, correspondent to ellagitannins (peak 1-punicalin, and peaks 9–12) and ellagic acid (peak 13), were also identified, together with some simple phenolic acids (gallic and hydroxybenzoic acids, peaks 2 and 4, respectively), digalloyl glucose (peak 5) and catechins (peaks 7 and 8) (Supplementary Figure S2, Table 3). Moreover, anthocyanins were detected only in UAE-ethanolic extracts of *P. granatum* fruit peels (TAC, Table 4).

Table 3. Peaks detected in the extracts of fruit peels of *P. granatum* by HPLC-ESI-Q-ToF analysis (peak numbers are referred to the chromatograms presented in Supplementary Figure S2).

Peak	Tr (min)	Raw Formula	Molecular Ion $[M-H]^-$ (*m*/*z*)	MS/MS Fragments	Peak Assignment
1	1.46	$C_{34}H_{22}O_{22}$	781.0564	600.9905, 450.9872, 301.0082	Punicalin
2	1.56	$C_7H_6O_5$	169.0142	n.d.	Gallic acid
3	1.71	$C_{48}H_{28}O_{30}$	1083.0594	n.d.	Punicalagin alpha
4	1.75	$C_7H_6O_3$	139.0384	n.d.	4-hydroxybenzoic acid
5	1.86	$C_{20}H_{20}O_{14}$	483.0874	331.0724, 313.0621, 271.0523, 169.0061, 125.0252	Digalloyl-glucose
6	1.96	$C_{48}H_{28}O_{30}$	1083.0597	781.0507, 600.9883, 300.9992	Punicalagin beta
7	2.05	$C_{15}H_{14}O_7$	305.0495	137.0233, 125.0187, 109.0478	(Epi)gallocatechin
8	2.65	$C_{15}H_{14}O_6$	289.0722	151.0119, 137.0278, 125.0178, 109.0235	Catechin
9	3.30	$C_{20}H_{16}O_{13}$	463.0525	300.9991	Ellagic acid-*O*-hexoside
10	7.20	$C_{20}O_{16}O_{12}$	447.0568	299.9915	Ellagic acid-*O*-rhamnoside
11	7.30	$C_{19}H_{14}O_{12}$	433.0419	299.9928	Ellagic acid-*O*-pentoside
12	7.91	$C_{14}H_6O_8$	300.9998	283.9960, 229.0127	Ellagic acid
13	9.80	$C_{21}H_{20}O_{11}$	463.0894	300.0278, 271.0258, 151.0035	Quercetin-3-*O*-glucoside

n.d.: not detected.

Table 4. Content of punicalagin alpha and beta, total anthocyanins content (TAC), and total polyphenol content (TPC) (in mg g^{-1} DW) in UAE-ethanolic and fermented extracts of pomegranate peels (*P. granatum*).

P. granatum	Punicalagin Alpha	Punicalagin Beta	TAC	TPC
UAE-ethanolic extracts	1.04 ± 0.09 ***	1.30 ± 0.15 ***	0.52 ± 0.07 ***	2.86 ± 0.34 ***
Fermented extracts	0.43 ± 0.03	0.53 ± 0.06	n.d.	0.96 ± 0.10

Results are given in mean ± SD ($n = 3$). Asterisks indicate significant differences between the content of the same compound class in different extracts (UAE vs. fermented), according to *t*-student test: *** $p \leq 0.001$, n.d.: not detected.

3.2. Antioxidant Capacity of the Extracts

The fruit extracts exhibited different antioxidant capacities depending on the extraction process applied (Table 5). For both species, the UAE-ethanolic extracts showed higher antioxidant activity (lower EC_{50} values) than the fermented ones ($p < 0.01$), regardless of the antioxidant method applied (DPPH or HRS). The UAE extracts showed to be around 2 to 5 times more effective as antioxidants than those obtained by fermentation. Moreover, *P. granatum* fruit-peel extracts exhibited the highest antioxidant potential (very low EC_{50} values), showing to be more than 100 times more antioxidants than the correspondent extracts of *S. nigra*.

Table 5. Antioxidant capacity (in terms of $EC_{50,}$ mg mL^{-1}) of UAE-ethanolic and fermented extracts of *S. nigra* fruits (elderberry) and *P. granatum* fruit-peels (pomegranate) extracts.

	EC_{50} Values	
	DPPH Assay	**HRS Assay**
S. nigra fruit extracts		
UAE-ethanolic extracts	0.632 ± 0.031 **	0.409 ± 0.031 ***
Fermented extracts	1.083 ± 0.082	1.672 ± 0.119
P. granatum fruit-peels extracts		
UAE-ethanolic extracts	0.004 ± 0.000 ***	0.024 ± 0.003 ***
Fermented extracts	0.018 ± 0.001	0.068 ± 0.004

EC_{50} values given in mean ± SD (n = 3). Asterisks indicate significant differences between the EC_{50} values of the different extracts (UAE vs. fermented) for the same antioxidant method, according to t-student test *** $p < 0.001$; ** $p < 0.01$.

4. Discussion

The content of polyphenols and the antioxidant capacity of the extracts obtained by fermentation and by UAE showed to be different, suggesting a higher efficiency of the UAE process compared to fermentation for both studied fruits. Indeed, an extraction process can be considered efficient if it allows obtaining a high content of the compounds of interest, also considering the principle of the Green Extraction, such as the amount and the type of solvent and the general procedure costs [44]. In particular, higher extraction efficiency decreases the time used during the process and increases the extraction yield [31]. Therefore, the UAE method applied here showed to be more effective, since used less time and temperature, and resulted in extracts with higher yields of polyphenols and antioxidant capacity, compared to those from the fermented-assisted extraction employed in this study. Based on the recent literature, this result was not predictable since the conversion of glucosides into their aglycones by fermentation may result in a higher antioxidant activity of these extracts and the fermentation of *Punica granatum* wastes utilizing *S. cerevisiae* may improve the extraction of ellagic acid derivatives [36]. In addition, the antioxidant activity of extracts obtained by fermentation cannot be hypothesized based on its TPC, since synergism between polyphenols and other components, sometimes deriving from the fermentation process, has been documented [45,46].

In agreement with our findings, flavonol glycosides (mainly quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside) and hydroxycinnamic acid derivatives have been described as major components in elderberry fruits [14,18]. Additionally, similarly to our results, rutin has been previously identified as the most abundant phenolic compound in elderberry extracts [47,48]. Caffeoylquinic acid derivatives and flavonols are used for the treatment of metabolic syndromes, being potential agents in the management of lipid metabolism and against obesity [49]. In addition, chlorogenic acid derivatives have shown antioxidant, anti-inflammatory, antibacterial, and antiviral properties [50,51], being important for cosmetic and pharmaceutical applications [52]. Moreover, quercetin derivatives are also important bioactive molecules, acting as antibacterial and antiviral [53], besides being important antioxidants. These flavonoids have also shown an anti-melanogenesis effect [54]. Both quercetin and hydroxycinnamic acid derivatives showed higher content in the UAE extracts than in those resulted from fermentation. This could be due to the duration of the fermentation process (24 h). Indeed, in a previous study using *Aspergillus niger*, the total amounts of polyphenols in *S. nigra* extracts were higher only if the fermentation time was longer than 24 h [55].

Regarding the anthocyanins, they were detected in higher amounts in fermented extracts of elderberry than in UAE ones. Previous reports showed that cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-glucoside, and cyanidin-3-sambubioside were the main anthocyanins found in extracts of *S. nigra* fruits [15]. The greater content in total

anthocyanins exhibited by the fermented extracts might contribute to their potential use as natural colorants, also with therapeutic effects [56].

Pomegranate peels are important sources of phenolic acids and hydrolyzable tannins [22,57], with punicalagin alpha and beta as the most abundant compounds [21,57,58]. In agreement with our results, Aguilar et al. [59] showed that the fermentation step decreased around six times the content of hydrolyzable tannins in *P. granatum* peels when compared to their content in fresh materials. Additionally, fermentation has already been shown to degraded punicalagin during the extraction of pomegranate [60].

The higher content of punicalagin in UAE ethanolic extracts observed here is an important result since punicalagin are the main compounds responsible for the in vitro and in vivo biological activity of pomegranate [61,62]. In addition, punicalagin point out as antioxidant molecules [27]. Therefore, the noticeable antioxidant capacity of the pomegranate fruit-peels extracts obtained by UAE in our study might be attributed to their richness in punicalagin. They also act in synergism with other compounds present in these extracts [58].

Although anthocyanins have already been described in peels of *P. granatum* [57], we did not detect these compounds in fermented extracts. These findings agree with the previous observation that the content of anthocyanins showed a significant decrease in pomegranate wine after fermentation [63].

The fermentation is known as a process that enhances the content of polyphenols in the extracts since the fermentation enzymes (e.g., amylases and xylanases) can release bound and non-soluble phenolics found in the wall matrix of the plant cells. In addition, the recent combination of an enzymatic pretreatment with microbial fermentation has been employed for the extraction of plant materials, including fruit peels, generating rich-polyphenolic products [64,65]. As such, this is a new extraction method that is being applied by several industries [33,35,66]. However, this method might also induce structural changes in polyphenols, increasing the rate of phenolics losses, and affecting their biological activities [33,35,66]. For instance, studies applying fermentation showed that oxidative degradation of polyphenols might occur, resulting in a low content of these compounds and weaker antioxidant capacity of the final extracts [35]. Thus, different extraction factors applied during the fermentation process (e.g., temperature, time, pH, and microorganism) should be carefully chosen and even optimized to generate extracts richer in polyphenols and avoiding hydrolysis and/or oxidation reactions of these bioactive compounds [35].

In conclusion, in our investigation, using the fruit of *S. nigra* and *P. granatum*, the ultrasound-assisted method showed to be more efficient than the fermentation process here tested to obtain extracts richer in polyphenols and with a greater antioxidant capacity. Indeed, although being more efficacious for the extraction of anthocyanins from elderberry fruits, the fermented-assisted method is a long process and might result in losses of bioactive compounds. These aspects, combined with the vast application, consolidation and robustness of the UAE method in different industries, as well as its ease-to-use and low cost, indicate that UAE could be a very promising method for both types of fruits to generate extracts with high polyphenolic content. Further studies, focused on the optimization of the parameters of both efficacious methods might be conducted, to develop new protocols more suitable and profitable for industrial applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7100386/s1, Figure S1: HPLC-ESI-Q-ToF analysis of fermented extracts of fruits of elderberry (*S. nigra*). Extracted Ion Chromatograms relative to the species listed in Table 1 (negative ionization mode). Figure S2: HPLC-ESI-Q-ToF analysis of fermented extracts of fruit-peels of pomegranate (*P. granatum*). Extracted Ion Chromatograms relative to the species listed in Table 3 (negative ionization mode).

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